Fibrinogen-Binding Proteins from *Staphylococcus aureus*.

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To my family, friends and colleagues.

CONTENTS

ABSTRACT

Staphylococcus aureus produces a large number of proteins that specifically bind to molecules from plasma or from the human extracellular matrix where they are involved in processes associated with the colonization of the host tissues. At least six different fibrinogen-binding proteins have been identified from *S. aureus*; Clumping factor A and B (ClfA and ClfB), Coagulase, Efb (previously Fib), FbpA , Map and Eap. This work focuses primarily on the function(s) of Efb and Eap.

We have shown that Efb contributes to the virulence of *S. aureus* in wound infection. An allele replacement mutant of Efb was constructed and compared with the virulence of the wild type in a rat model of wound infection. Sixty-seven % of the animals challenged with the parental strain developed severe clinical signs of wound infection, whereas only 29 % of the rats infected with the isogenic mutant showed severe symptoms. The function of Efb in infection is unclear but we have shown in vitro that multiple binding sites are involved in the interaction between Efb and fibrinogen. One binding site is located at the C terminus of Efb and one binding site at the two repeat regions of the N terminus. These N-terminal repeats are homologous to those at the C terminus of coagulase. The divalent binding nature of Efb with Fg leads to the precipitation of the Efb-fibrinogen complex, which can be enhanced by Ca^{2+} or Zn^{2+} but not by Mg^{2+}. At least one of the binding sites for Efb is located at the Aα-chain of Fg (Aα 111 - Aα 197). Binding of Efb to this site may serve to block specific Aα-chain processes. In addition, we have shown that Efb could bind to fibrin as well as to fibrinogen and that the two repeats of Efb could compete with the five repeats of coagulase for the same binding domain on fibrinogen.

We have shown in this study that Eap (extracellular adherence protein) have the ability to enhance the adherence of *Staphylococcus aureus* to host cells. Eap can bind at least seven plasma proteins, including fibrinogen, prothrombin and fibronectin. Eap is presumably the same, or at least related to a previously described cell surface protein, designated Map for major histocompatibility complex class II analogous protein. In contrast to Map, Eap is mainly extracellular since 70% of the protein was found in the culture supernatant of *S. aureus* strain Newman. In this study we proposed a novel mechanism for adherence of *S. aureus* to host components stimulated and mediated by Eap. This is based on the fact that; i) Eap can form olimeric forms ii) *Staphylococcus aureus* strain Newman adhere to immobilized and soluble Eap, but not to the other staphylococcal proteins tested iii) Eap was able to cause agglutination of *Staphylococcus aureus* and iv) Attachment of *S. aureus* to fibroblasts and epithelial cells was significantly enhanced by the presence of Eap.
In conclusion, *Staphylococcus aureus* produce several fibrinogen-binding proteins that interact with fibrinogen in different ways. Colonization of the host tissues and dissemination of the infection is a multifactorial event in which many extracellular and cellsurface proteins are involved. FgBPs play an important role during infection by: a) A cellsurface FgBP that mediates the direct attachment of *S. aureus* to damaged heart valves and implanted biomaterial, b) A secreted FgBP with broad binding specificity that promotes colonisation of host tissues and bacterial aggregation, and c) A 15.9 kDa secreted FgBP that influences the severity of a wound infected by *S. aureus* thereby altering the healing process.

MAIN REFERENCES

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I **Palma, M., Nozohoor, S., Schenning, T., Heimdahl, A., and Flock, J. - I.** (1996). Lack of the Extracellular 19-Kilodalton Fibrinogen-Binding Protein from *Staphylococcus aureus* Decreases Virulence in Experimental Wound Infection. *Infect Immun*. **64**, 5284 - 5289.

II **Marco Palma, David Wade, Margareta Flock, and Jan-Ingmar Flock.** (1998). Multiple Binding Sites in the Interaction Between an Extracellular Fibrinogen-Binding Protein from *Staphylococcu aureus* and Fibrinogen. *J. Biol. Chem*. **273** (12) : 13177 - 13181.

III **David Wade, Marco Palma, Ingered Löfving-Arvholm, Matti Sällberg, Jerzy Sillberring, and Jan-Ingmar Flock.** (1998). Identification of Functional Domains in Efb, a Fibrinogen-Binding Protein of *Staphylococcus aureus*. *Biochemical and Biophysical Research Communications*. **248**, 690-695.

IV **Marco Palma and Jan-Ingmar Flock.** (1999). The D Fragment of Fibrinogen is Recognized by an Extracellular Fibrinogen-Binding Protein, Efb, from *Staphylococcus aureus*. Manuscript

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ABBREVIATIONS

ECMBP	Extracellular matrix binding proteins
FgBPs	Fibrinogen-binding proteins
Efb	Extracellular fibrinogen-binding protein
Clf	Clumping factor
Eap	Extracellular adherence protein
Map	Major histocompatibility complex class II analogous protein
FnBPA	Fibronectin-binding protein A
FnBPB	Fibronectin-binding protein B
Cna	Collagen-binding protein
Fg	Fibrinogen
Fn	Fibronectin
Vn	Vitronectin
Pt	Prothrombin
Cn	Collagen
Pl	Plasmin
MRSA	Methicillin-resistant *S. aureus*
FpA	Fibrinopeptides A
FpB	Fibrinopeptides B
FPLC	Fast protein liquid chromatography
InlA	Internalin A
InlB	Internalin B
NVE	Native valve endocarditis

INTRODUCTION

General background about S. aureus

The family Micrococcaceae consists of four genera: Planococcus, Stomatococcus, Micrococcus and Staphylococcus the latter of which is the only genus of medical importance in this family. Staphylococci which infect humans include 13 coagulase - negative species (CNS) including *S.epidermidis, S. capitis, S. saccharolyticus, S. warneri, S. haemolyticus, S. hominis, S.lugdunensis, S. schleiferi, S. auricularis, S. saprophyticus, S. cohnii, S. xylosus and S.simulans* and one coagulase - positive species, *S. aureus*. Other species of coagulase positive staphylococci have been recognised in different mammals as *S. intermedius* in dogs, *S.hyicus* in pigs and *S. delphini* in dolphins (48).

Staphylococci are non-motile, Gram-positive, catalase-positive, facultative anaerobic cocci usually arranged in irregular clusters. Robert Koch was the first to describe staphylococci in human pus. Later Alexander Ogston introduced the name *Staphylococcus* from the Greek word staphyle meaning ”bunch of grapes” (70). Two staphylococcal species were discribed by Rosenbach in 1884 based on the pigmentation of the colonies (79): *Staphylococcus aureus* with golden yellow colonies and *Staphylococcus albus* with white colonies, whose name was later changed to *Staphylococcus epidermidis*.

Diseases caused by S. aureus

Today, 49 years after the introduction of penicillin, staphylococci continue to plague humans and animals. This microorganism under certain circunstances can easily be converted to a pathogen which infects immunocompromised patients as well as healthy individuals causing both hospital- and community acquired infections. With its broad tropism for host tissues, this pathogen can colonize many different parts of the body (heart valve, lungs, skin and bones) demonstrating adaptibility and ability to disseminate.

The list of diseases caused by *Staphylococcus aureus* is long (Table I) and includes a range of infections from soft tissue infections to severe, life threating debilitations. The most simple infection, folliculitis, is a pyogenic infection localized to the hair follicle. Staphylococcal wound infections often follow surgery or traumatic injuries. In USA, 25% of nosocomial infections are post-operative wound infections, 20-30% of which are caused by *S. aureus* (27). From local skin infections, the organism can enter the blood stream causing septicemia. Septicemia can be a serious condition, often acquired in the hospital either in association with implanted medical devices or during post-operative recovery.

Staphylococci in septicemia are easily disseminated causing metastatic infections such as osteomylitis. Osteomylitis is an infection of the bone which can result from hematogenous spreading of bacteria or from the direct introduction of microorganism from external sources as in trauma. *S. aureus* is the most frequent cause of osteomyelitis. In children the infection involves the metaphyseal area of the long bones, whereas osteomyelitis in adults commonly involves the vertebrae of the spine.

Another type of staphylococcal dissemination is infective endocarditis which is an infection of a heart valve usually characterized by a vegetation on the aortic valve. Acute endocarditis caused by *S. aureus* is a serous disease with a mortality rate of 50 %. *S. aureus* is responsible for about 30 percent of cases of native valve endocarditis (NVE) and 50% of endocarditis in intravenous drug users whose disease normally involve the tricuspid valve at the right side of the heart. Rheumatic fever, congenital cardiac defect, degenerative valvular disease, previous endocarditis and prosthetic valves are predisposing factors for infective endocarditis.

Table I. Diseases caused by *Staphylococcus aureus*.

INFECTION	**HOST TISSUE**	**INTOXICATION**	**HOST TISSUE**
Wound infections	Skin	Food poisoning	Intestinal epithelial cells
Folliculitis	Hair follicle	Scalded skin syndrome	
Septicaemia	Blood	Toxic shock syndrome	
Endocarditis	Heart valve		
Osteomyelitis	Bone		
Arthritis	Joint		
Pneumonia	Lung		
Mastitis	Mammary glands		

S. aureus can also cause infections in animals. For example, this pathogen is the leading cause of intramammary infection in cattle and is the most economically important disease in the dairy industry. Mastitis accounts for approximately 70% of the total expenses for dairy farms resulting in the loss of billions of dollar in the USA. In addition, *Staphylococus aureus* can cause pneumonia, impetigo, food poisoning and toxic shock syndrome.

A major problem in the treatment of staphylococcal disease has been the increase of methicillin-resistant *S. aureus* (MRSA) strains in many countries including Japan, Denmark

and countries of southern Europe. After the introduction of methicillin in the 1960's, the incidence of MRSA has grown significantly. The most likely explanation for this appearance and spread of MRSA is the increased use and abuse of broad spectrum antibiotics. Certain strains of MRSA, the so called multiresistent types, not only have developed resistance to methicillin but also to most other antibiotics with the exception of vancomycin. The future emergence of vancomycin-resistant strains could be disastrous. Consequently, rapid diagnosis, restricted use of antibiotics and new alternative treatments are urgently needed.

Virulence factors associated with S. aureus

Staphylococcus aureus produces virulence factors that participate. i) in the bacterial attachment to host components, ii) in the evasion of host defences and iii) the spread to distal or deeper tissues.

i) *Staphylococcus aureus* attaches to components of the cell surface or to the extracellular matrixes as one of the first steps in the colonization of the host tissues. A family of adhesins called ECMBP (extracellular matrix binding proteins)(Table II) recognize specific host matrix proteins such as fibronectin (Fn) (21, 39), collagen (Co) (74, 89, 90), elastin (56, 72) and bone sialo protein (80). Certain plasma proteins including fibrinogen (Fg) (9, 60), prothrombin (Pt) (10, 77), vitronectin (Vn) (55, 75), thrombospondin (33) and plasminogen (Pl) (50, 81) are also recognized. Most ECMBP are attached to the staphylococcal cell wall by a common mechanism, the LPXTG sequence. LPXTG is cleaved between threonine and glycine by sortase. The threonine is then covalently linked to a pentaglycine bridge of uncross-linked peptidoglycan. However, cell surface associated Map and elastin-binding protein lack the LPXTG motif indicating that there are also other mechanisms in gram positive bacteria that anchor the protein to the cell wall.

The most well characterized of the ECMBP are the two fibronectin-binding proteins (FnBPA and FnBPB), collagen-binding protein (Cna) and clumping factor (Clf). The FnBPs mediate colonization to various sites in the body where fibronectin is present especially in damaged heart valves and implanted medical devices. Studies with double mutants in the *fnb*A *fnb*B genes indicate that fibronectin-binding is important in bacterial attachment to biomaterials which have been in contact with the blood (26). Other adhesins such as FgBP seem to be important in the adherence to biomaterial which have been in contact with the blood for less than 60 minutes (93). Discrepant results have been reported where a FnBP defective mutant was studied in an animal model of infective endocarditis. One of study demonstrated that the isogenic mutant reduced adherence to valvular vegetations (52). However these results were not replicated in a second study (22). Collagen recognition in *Staphylococcus aureus* is mediated by a surface collagen-binding protein called Cna. That collagen-binding plays a role in infection was demonstrated in a septic arthritis model using a mutant deficient in Cna (73).

A Clf surface-associated protein is responsible for direct binding of staphylococcal cells to fibrinogen and fibrin (see the special section about FgBPs).

Table II. List of ECMBPs from *Staphylococcus aureus*

ADHESIN	MW (KDA)	HOST MOLECULE	REFERENCES
ClfA ClfB	92 (190) 124	Fibrinogen	McDevitt *et al*., 1992 Ní Eidhin *et al*., 1998
Map	72 or 60	Fibrinogen Fibronectin Vitronectin Elastin.	Homonylo McGavin *et al*., 1993
FbpA	70	Fibrinogen	Cheung *et al*., 1995
FnBPA FnBPB	108 98	Fibronectin	Jönsson *et al*., 1991 Greene *et al*., 1995
Cna	135, 110	Collagen	Switalski *et al*., 1989, 1993
VnBP	60	Vitronectin Heparin	Liang, Flock & Wadström, 1995
Bone sialo protein -binding protein	97	Bone sialo protein	Rydén *et al*., 1989
Thrombospondin-binding protein		Thrombospondin	Hermann *et al*., 1991
EbpS	44	Elastin	Park *et al*., 1991, 1996
Staphylokinase		Plasminogen	Sako *et al*., 1983
Laminin-binding protein	52	Laminin	Lopes, dos Reis & Lowy, 1996

ii) *Staphylococcus aureus* produces factors that are important in the microorganisms defense against antibodies and phagocytosis. Several proteases from this organism such as the serine protease V8 have the ability to cleave and inactivate IgG and thus have a direct effect on specific antimicrobial peptides like the neutrophil defensins.

Another group of proteins that participate in staphylococcal defence are the superantigens (including enterotoxins A to E, TSST-1 and the exfoliative toxin A and B). These superantigens interfere with the immune process by binding to major histocompatibility complex class II (MHC II) and the variable region of the T-cell receptor β-chain. This activates T - lymphocytes expressing a particular T-cell receptor, leading to the release of relatively large quantities of cytokines. Superantigens are antigens that do not require phagocytic processing. It is likely that these toxins prevent the host from producing antibodies to staphylococcal antigens during the course of an infection.

Protein A is a cell surface protein that binds to the Fc portion of IgG antibodies such that the antibodies that are misaligned thus interfering with the binding of phagocytic cells to IgG. Staphylococcal cells coated with antibodies evade the immune response and prevent opsonization. This was was confirmed using a protein A deficient mutant (Spa^-) which showed that Spa^- was taken up more efficiently by polymorphonuclear leucocytes than was the parental strain (25). Complementary data demonstrated that Spa^- mutant was less virulent than the wild type in a murine model of infection (23).

The role of coagulase is unclear but it has been shown that this protein converts fibrinogen to fibrin forming a fiber layer around the bacteria. This layer probably shilds the organisms from phagocytosis.

S. aureus is generally not considered to be an intracellular pathogen, but previous reports have shown that *S. aureus* cells can be internalized in nonprofessional phagocytes such as epithelial- (1) and endothelial cells (28). Bacteria induce internalization via a mechanism involving membrane pseudopod formation and the escape into the cytoplasm following lysis of the endosomal membrane. Furthermore, internalization of *S. aureus* can induce apoptosis in epithelial cells (7). Internalization provides protection against host defenses and antibiotic treatment, but how internalization occurs and which staphylococcal factors are involved is still unclear.

iii) *Staphylococcus aureus* can secrete several proteins that facilitate the spread of the microorganism through tissue. Hemolysins are a group of molecules (α-, β-, δ-, γ- toxins) that can damage the cytoplasmic membrane of the eukaryoric cells. For instance, α-toxin (alpha-haemolysin) can lyse several cells, including erythrocytes, leukocytes, hepatocytes, platelets and human diploid fibroblasts. Site directed mutagenesis of the *hla* gene has been used to study α-toxin.

Hyaluronidas is an enzyme that digests hyaluronic acid, a component present in the matrix of connective tissues. This protein which is produced by 95-100 % of coagulase positive staphylococci and was previously referred to as a spreading factor.

The list of staphylococcal virulence factors is long. Staphylokinase, catalase, lipases, penicillinases, Toxic shock syndrome toxin-1 and exfoliative toxins are only a few exemples.

Fibrinogen-binding proteins from S. aureus

Staphylococcus aureus interacts with fibrinogen through both secreted and surface proteins. At least six different fibrinogen-binding proteins have been identified from *S. aureus* so far; Clumping factor A and B (ClfA and ClfB), Coagulase (Coa), Efb (previously Fib), FbpA (surface-associated coagulase), Map and Eap (Table III).

Extracellular fibrinogen-binding protein (Efb)

Extracellular fibrinogen-binding protein, or Efb (previously known as Fib (9)) is a 15.9 kDa fibrinogen-binding protein secreted by *Staphylococcus aureus*. Early studies showed that the *efb* gene and its gene product were found in all strain of *S. aureus*, but absent in other staphylococcal species such as *S. epidermidis, S. hyicus, S. lugdunensis and S. intermedius*, indicating that Efb is unique to *S. aureus* (12). Efb consists of 136 amino acids with no cystein residues and contains two nearly identical 22 amino acid segments at the N - terminal region which are similar to the fibrinogen-binding domain of coagulase (11)(Figure 1). No LPTXG amino acid sequence is found in Efb; a motif that is required for attachment of surface localized proteins to the cell wall. The predicted isoelectric point of 10.1-10.3 is due to its high lysine content which is about 14%.

A mouse mastitis model was used to investigate the effect of immunization with Efb and collagen-binding protein (Cna) against the staphylococcal infection. Mice vaccinated with FgBPs showed a reduced rate of mastitis compared to controls vaccinated with CnBP. Furthermore, no pathological changes of the mammary glands were observed in the group vaccinated with FgBPs compared with an unvaccinated group. In addition, the number of bacteria recovered from the glands of the infected mice was significantly lower after FgBP immunization (57).

In a recent study the antibody response to Efb was measured in patients with various staphylococcal infections (17). The antibody titer in the acute phase was significantly lower than the normal population. In patients with arthritis and osteitis, the antibody response during the convalescent phase increased by 67 % compared to controls. In patients with absceses, a 50% increase above control levels was noted. The elevated response indicates that Efb is expressed and secreted in vivo by *Staphylococcus aureus*.

Clumping factor.

In 1908, Much (64) observed that staphylococcal cells from clinical isolates undergo clumping in the presence of plasma and easily form visible aggregations. Fibrinogen is unique among the plasma proteins in being able to stimulate clumping in certain strains of *Staphylococcus aureus*. It was originally believed that the clumping factor was coagulase

which is situated on the bacterial cell surface. But later it was demonstrated that the agglutination of staphylococcal cells is associated with the cell wall protein clumping factor

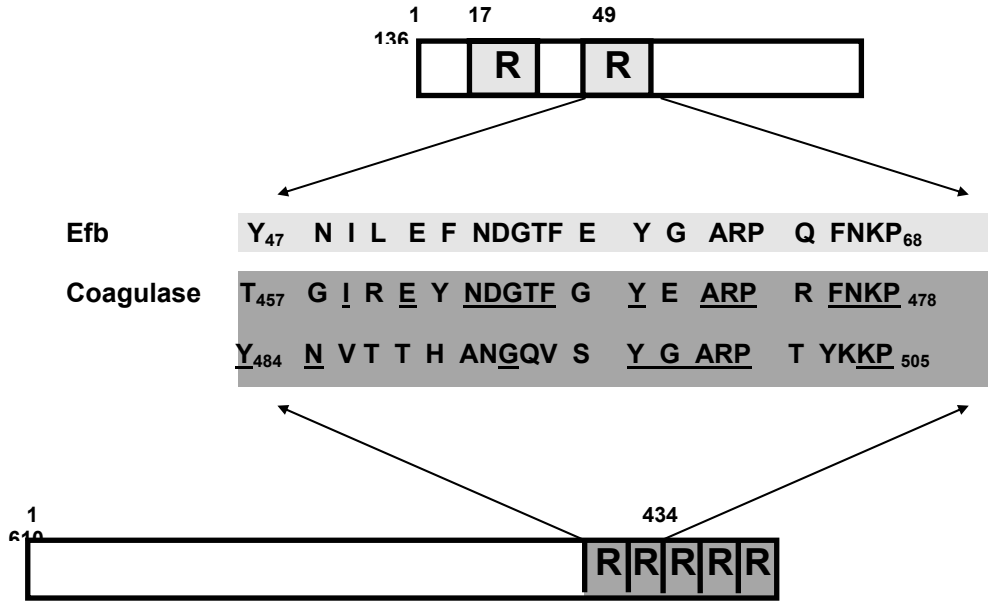


Figure 1. Schematic drawing of coagulase and Efb. The second repeat of Efb has high homology with the repeats of coagulase.

(Clf). That this is not the case was demonstrated using an allele replaced *S. aureus* mutant defective in the coagulase gene (62). This mutant was deficient in coagulase activity but still interacted with fibrinogen indicating that the bound form of coagulase is not responsible for clumping and for the adherence of *Staphylococcus aureus* to solid phase fibrinogen.

The high affinity of fibrinogen for staphylococcal clumping factor was shown in earlier studies. At this time it was estimated that 2130 fibrinogen molecules bound with a dissociation constant (K_d) of 9.9 nM to a single staphylococcal cell from strain Newman (32). But further characterization of the interaction between a fusion protein that contains the fibrinogen-binding domain of ClfA and an immobilized fibrinogen using a BIAcore biosensor yielded a K_d of 0.51 ±0.19 μM (61). The differences of these affinity values can be explained in two ways: i) in the first study, *S. aureus* strain Newman expressed several FgBPs that can be found on the cell surface and may contribute to adhesion to fibrinogen, and ii) in the second study, fibrinogen coupling to chip lost its structure and binding capacity.

A mutant defective in the fibrinogen receptor was isolated by transposon Tn917 mutagenesis in *S. aureus* strain Newman (60). Using this approach, McDevitt and co-workers cloned and characterised the gene (*clfA*) which encoded the protein responsible for the clumping reaction. The ligand-binding domain of ClfA is a 218-residue segment located between residues 332-550 in Region A (60). This region recognizes the amino acid sequence GAKQAGDVHPAETEYDSLY (46, 88) in the carboxyl-terminal of the γ-chain of the fibrinogen monomer. This region corresponds to the region that binds to GPIIb-IIIa receptor on platelets (31). A potential calcium binding EF-hand like motif was identified between residues 310 and 321 of Region A. (69). In addition, a high degree of similarity was found between a region in the fibrinogen-binding site of ClfA which displays a cation-binding motif, (MIDAS motif) located in the I (A) domain of integrins. The MIDAS motif which binds Mg^{2+} helps to determine the correct structure of the binding domain and thus facilitates the interaction of ClfA with fibrinogen. Deletion mutagenesis and site-directed mutagenesis in both the EF-hand like motif or in the MIDAS motif resulted in a significant reduction in the affinity for native fibrinogen and for a fibrinogen γ-chain peptide. These data suggest that the ClfA protein interacts with fibrinogen through a mechanism similar to the platelet integrin.

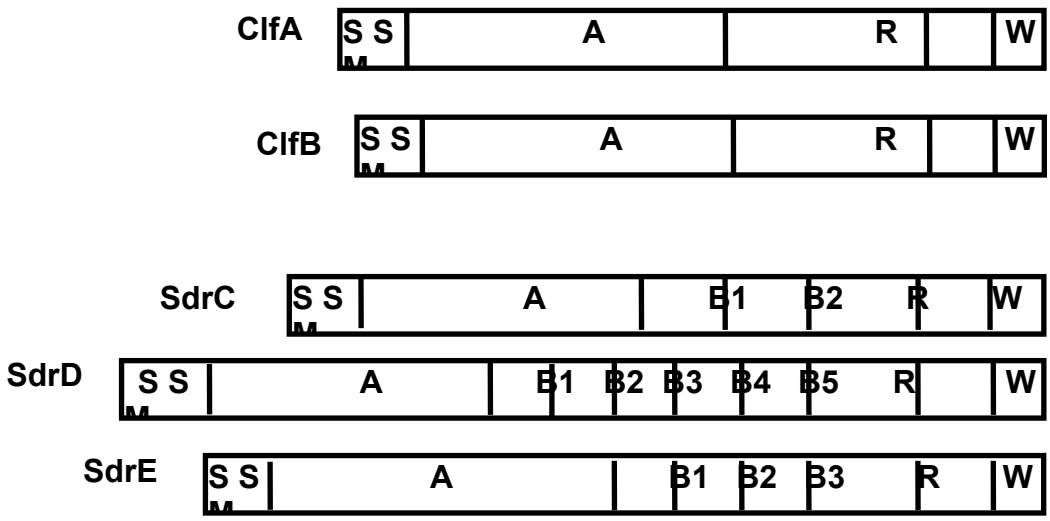


Figure 2. Structural organization of ClfA, ClfB, SdrC, SdrD and SdrE. SS, signal sequence; A, unique nonrepetitive sequence; B, B-repeats with the EF-hand loops; R, repeated domains containing Ser-Asp residues; W, cell wall-spanning domain; M, hydrophobic membrane-spanning domain (Josefsson *et al*., 1998).

A second clumping factor, ClfB, which is encoded by a distinct gene (*clfB*) was recently demonstrated in *S. aureus* (67). The domain organization of ClfB is similar to ClfA including a signal sequence (region A), region R which is composed of Ser-Asp repeats, a peptidoglycan spanning region (W), an LPTXG motif, a hydrophobic membrane-spanning region and a short positively charged C-terminus (Figure 2). The degree of sequence homology between ClfA and ClfB is 41% for the signal region, 36% for the anchoring domain and 26% for region A. The diversity in the A region may explain why ClfB recognizes a different domain on Fg than that which is recognized by ClfA. Furthermore, ClfB binds to the Aα- and Bβ-chains of Fg as opposed to ClfA which binds to the γ-chain. ClfB is only detectable during the early exponential phase of the bacterial growth compared with ClfA which is present at all stages. ClfB promotes binding to immobilized Fg and clumping only during the exponential-phase of the growth. ClfB like ClfA binds Ca^{2+}. ClfB also has a MIDAS-like motif as well, however, it is located in a different position than the MIDAS-like motif on ClfA.

In addition, a new subfamily of proteins from *S. aureus* (the Sdr subfamily) which function as clumping factors have recently been described (37) (Figure 2). Three members of this subfamily, SdrC, SdrD and SdrD, have been characterized and shown to have organizational and sequence similarities to ClfA and ClfB. But in constrast to ClfA and ClfB, Sdr proteins have an additional motif, a B-motif, located between region A and region R. Each B-motif is composed of 110-113 residues with the number of motifs varying between 3 and 5 among the Sdr proteins. EF-hand motifs which bind Ca^{2+} have been found in each B-motif and are critical for the conformation of the Sdr protein. Furthermore, the amino acid sequence of region A of the Sdr proteins have only 20-30% homology with the same region on ClfA and ClfB suggesting different binding properties and function compared to ClfA and ClfB.

Clumping of *S. aureus* in plasma and staphylococcal adherence to fibrinogen substrata has been suggested as important virulence factors. Evidence that ClfA can contribute to the pathogenesis of infection associated with implanted medical devices and with infective endocarditis has been presented (IE) previously (63). The biological role of ClfA in vivo was demonstrated in a study where a clumping factor defective mutant was compared with the parental strain. This study showed that the mutant had up to 100 times lower affinity for fibrinogen and produced 50 % less endocarditis in rats with catheter-induced aortic vegetations compared to the parent strain. The infection rate was restored to that of the wild-type when a copy of the wild type gene was inserted into the *clfA* mutant. In other studies, this mutant showed reduced adherence to blood-conditioned catheters that had been in contact with the host for period of less than one hour (93).

Fibrin is the major component of blood clots and fibrinogen is one of the major plasma proteins deposited on surface of implanted biomaterial (94). Several blood components, including fibrinogen have been implicated in promoting bacterial attachment to implanted materials. Fibrinogen probably initiates staphylococcal adherence to foreign surfaces. But the adsortion of this molecule is only transient and is replaced by other plasma proteins at a later stage after Fg has been proteolytic degraded by plasmin (93).

Coagulase

The protein, Coagulase, is produced by *Staphylococcus aureus* which stimulates fibrinogen polymerization and the clotting of plasmas in several mammalian species. Coagulase exerts its biological effects through the activation of prothrombin via a non enzymatic process. In this process, staphylocoagulase acts as an allosteric effector that changes the conformation of prothrombin in such a way that active binding centres are made accessible to fibrinogen (44). Functional studies with α-chymotrypsin generated fragments of coagulase have identified the biologically important regions of the protein involved in prothrombin-binding and the coagulase activity (43, 45). A 43 kDa fragment containing 324 amino acids located in the NH_2 -terminal portion posesses both prothrombin and coagulase activity. This fragment binds strongly to the C-terminal region of human prothrombin (44) with a dissociation constant of 1.7 nM compared to the intact staphylocoagulase of 0.49 nM. Staphylocoagulase is classified into eight different serotypes based on variations in the N-terminal sequence (92). Variations in this region are related to the different affinities of coagulase for prothrombin.

In addition to prothrombin-binding, coagulase can interact directly with fibrinogen (10). The region required for prothrombin- and fibrinogen-binding are located on different parts of the coagulase molecule. A 177 residue segment of the carboxy terminal of coagulase has been implicated in fibrinogen-binding as shown in a study where this fragment was fused to β-galactosidase (62).

The predicted molecular weights of the extracellular forms of staphylocoagulases vary depending on the number of tandem repeats of a 27-residue sequence at the carboxy terminus. For example, strain 8325-4 has a 69 kDa coagulase with five repeats (77), strain 213 has a 72 kDa protein with six repeats (40) and strain BB 77 kDa protein with eight repeats (41). The 87 kDa coagulase from strain Newman seems to be identical to the coagulase from strain 8325-4, but its binding to prothrombin has not been established.

Staphylocoagulase is mainly an extracellular protein but a certain amount can be found on the surface of staphylococci as well. A previous study demonstrated that a small population of coagulase molecules remain tightly bound to the staphylococcal cell wall. There is now evidence that staphylocoagulase exists in multiple forms. Thus, not only different strains

produce different forms of staphylocoagulase but the same strain may produce multiple forms.

Table III. FgBPs from *Staphylococcus aureus*.

PROTEIN	LOCATION	MW (KDA)	REFERENCES
ClfA	Cell surface	90(190)	McDevitt *et al*., 1992
ClfB		125	Ni Eidhin *et al*., 1998
FbpA	Cell surface	70	Cheung *et al*., 1995
Map	Cell surface	72 or 60	Homonylo McGavin *et al*., 1993 Jönsson *et al*., 1995
Efb (Fib)	Extracellular	15,9	Bodén & Flock, 1989, 1994 Palma *et al*., 1998 Wade *et al*., 1998
Coagulase	Extracellular	87	Bodén & Flock, 1989
Eap	Extracellular	60	Palma, Haggar & Flock, 1999

The role of coagulase in virulence is unclear. Previously studies suggested that in vivo coagulase stimulates dissemination, intra-vascular coagulation and enhances tissue survival of cocci by inhibiting phagocytosis. Several authors have investigated the action of staphylocoagulase in vivo and determined the intravenous lethal dose of staphylocoagulase for rabbits and mice (19). The pathological changes induced by purified staphylocoagulase in the laboratory animals was also studied previously (2). Several reports have shown that coagulase deficient mutants are less virulent in a mouse model of infection (29, 36). The problem with these experiments is that the mutants were isolated after chemically-induced mutagenesis. Thus, a loss of other possible virulence determining factors must be considered. A later study which compared the virulence of a site-specific, coagulase-deficient mutant with the coagulase-positive parental strain DU 5808 in a rat endocarditis model concluded that there was no differences in virulence between these two strains (5). These data support previous results from a murine model of infection and mastitis which indicated that coagulase is not a virulence factor (77). However, it should be considered that some virulence factors may play important roles only in specific infections and probably have different functions in different infections. Recent studies on coagulase-deficient mutants show that coagulase is important in hematogenous pulmonary infections (82). When mice are injected intravenous with *Staphylococcus aureus* enmeshed in agar beads, multiple lung abcesses surrounding the

pulmonary arterioles form. These abcesses consisted of bacterial colonies encircled by fibrin filaments and surrounded by neutrophils and macrophages. Animals infected with the coagulase-deficient strain or other bacterial species did not display these symptoms.

FbpA

Cheung *et al* cloned and sequenced the gene for a cell surface fibrinogen-binding coagulase (FbpA) (16). The sequence of a 34 kDa identified protein was identified containing a unique stretch of 11 amino acids between residues 409 and 419 (SVTLPSITGES) which shares homology with a cell wall anchor motif (LPXTGX).

Major Histocompatibility complex class II analogous protein (Map)

Map was first described by Homonylo McGavin *et al* as a 72-kDa surface protein that was capable of binding to fibrinogen and several other extracellular matrix proteins, including fibronectin, bone sialoprotein, vitronectin and thrombospondin (34). Later the gene coding this protein was cloned and sequenced (38). Map consists predominantly of six repeated domains of 110 residues each. Each repeated domain contains a subdomain of 31 residues that share striking sequence homology with a segment in the peptide binding groove of the β chain of major histocompatibility complex (MHC) class II protein in different mammalian species. The interaction effects of Map and MCH II in infection are unknown.

Influence of agr on FgBPs

Very little is known about the regulation of the different FgBPs in *S. aureus*. Previous studies by Cheung and coworkers demonstrated that *coa* transcription was negatively modulated by *agr* and occurred mainly during the exponential growth phase. In contrast, *clfA* trancription was *agr* independent and was strongest during the postexponential phase (97). A triple mutant (*agr*, *coa* and *clfA*) showed decreased binding to Fg than the double mutant (*agr* and *coa*), but still retained some binding capacity. These data indicate that additional fibrinogen-binding components may be expressed such as ClfB, FbpA, Map, Eap or Efb.

Fibrinogen-binding proteins from microorganisms other than S. aureus

Fbe from S. epidermidis

Staphylococcus epidermidis is an important cause of infections associated with medical devices as in prosthetic valve endocarditis, infections related with catheters or artificial joints. Immediately after implantation, biomaterials are covered with plasma proteins including fibrinogen which are targets of several strains of *S. epidermidis* (101). Baldassarri and coworkers reported that *S. epidermidis* slime-negative strains adhered significantly better to Fg than the slime positive strains (6). A fibrinogen-binding protein from *S. epidermidis* (Fbe)

has been identified on the surface of the bacterium. Phage particles that bound specifically to immobilized fibrinogen were selected from a phage display library containing chromosal DNA from *S. epidermidis* (68). Fbe has the LPXTG motif and the predicted charged wall region usualy found in cell surface proteins on Gram positive bacteria. Some similarity has been noted between the A region of ClfA and ClfB and the region containing residues 295 to 545 of Fbe. Furthermore, Fbe interacts with the β-chain of fibrinogen and Fbe-Fg binding can be stimulated by physiological concentrations of Ca^{2+} (76).

M protein from S. pyogenes

Streptococcus pyogenes is an important cause of pharyngitis, scarlet fever, streptococcal toxic shock syndrome, erysipelas, pyodermas and nonsuppurative sequelae such as acute rheumatic fever and glomerulonephritis. *S. pyogenes* recognizes and binds to plasma fibrinogen (51) with 8000-10000 fibrinogen-binding sites per bacterial cell (35). This interaction is associated with M protein (42), a fibrous protein located on the surface of the bacteria.

The region located near the tips of M protein binds to the D fragment of Fg (96) with a dissociation constant of 10^{-9} to 10^{-7} M (35). M protein is an important virulence factor of *S. pyogenes* because of its antiphagocytic capacity. Fibrinogen binds and covers streptococcal cells which inhibit the complement deposition on the bacterial surface, this allows the bacterium to evade opsonization (20).

Other streptococcal components such as T protein (83) and G3PDH (71) also bind to Fg in a manner similar to M protein (86).

Proteases from Bacteroides

Bacteroides gingivalis and *Bacteroides intermedius* have been identified as causes of human periodontal diseases. Infections of this type are characterized by gingival inflammation accompanied by a loss of connective tissue and bone from around the roots of the teeth. *P. gingivalis* interacts with some Gram-positive bacteria such *Actinomyces viscosus* (84), *Streptococcus gordonii* (87), and S*treptococcus oralis* (66). This interaction may play an important role in the attachment and colonization of *P. gingivalis* to periodontal sites. It has been reported that fibrinogen is the most active inhibitor of *P. gingivalis* and *S. oralis* coaggregation. The inhibitory factor produced by Fg has been localized to residues 158-176 and 192-206 of the Aα-chain of fragment D which contains four and two arginine (65). Two different fibrinogen proteinases, Lys-gingipain (gingipain-K) and Arg-gingipain (gingipain-R) have been identified in *P. gingivalis*. These proteinases degrade Fg so as to inhibit clot formation and thus increase bleeding at periodontitis sites.

Bacteroides gingivalis and *Bacteroides intermedius* bind with high affinity (K_d of 10 to 30 nM respectively) to different sites on fibrinogen (54). A 150 kDa cell surface protein from *B. gingivalis* recognises a region of fibrinogen located between the D and E domains and proteolytically degrades fibrinogen into two major components of 120 and 150 kDa (53).

Cell surface protein from Candida

Candida albicans is a part of the normal human flora but can cause opportunistic infections in immunocompromised hosts. It has been reported that this microorganism can also recognize human fibrinogen. A 68 kDa protein from the cell surface of this organism mediates attachment to the D-domain of Fg. The average number of binding sites per cell is estimated to be approximately 6000 with a dissociation constant of 5.2×10^{-8} M (3).

Fibrinogen

Fibrinogen, the precursor of fibrin, is a 340 kDa plasma glycoprotein which plays a major role in the process of blood clotting. Fibrinogen is arranged symetrically into three globular domains with one central domain (E) and two distal domains (D)(Figure 3). Each fibrinogen molecule consists of three pairs of non-identical polypeptide chains (AαBβγ) arranged so that all six amino - termini are located in the central part of the molecule (18). The Aα-chains consists of 610 residues and has a molecular mass of 67 kDa. The Bβ-chain is a 55 kDa polypeptide composed of 461 residues and the 48 kDa γ-chain has 411 residues. Aα–, Bβ– and γ-chains are linked together in the central amino-terminal domain by three interchain disulfide bridges.

Tissue injury results in disruption of blood vessels which leads to the exposure and interaction of blood components with subendothelial structures. This triggers a series of reactions in which fibrinogen participates in the final stage. Thrombin cleaves Arg-Gly bonds in fibrinogen between Aα16 and 17 and between Bβ14 and 15 releasing fibrinopeptides A (FpA) (ADSGEGDFLAEGGVRGPRV) and B (FpB)(XGVNDNEEGFFSARGHRP), respectively (8). Fibrinopeptide A is released significantly faster than fibrinopeptide B under typical conditions. Only FpA is required for clot formation whereas FpB is chemotactic for human polymorphonuclear neutrophils and fibroblasts (85). FpA and FpB release exposes a binding site (49) in the E domain of Fg which subsequently aligns with a complementary site in the D domain of another molecule. The clot matrix is stabilized by the formation of covalent crosslinks between different molecules. Factor XIIIa which is a plasma transglutaminase catalyses the formation of isopeptide bonds between the γ-chains (15) and links the lysine at position 406 with a glutamine at position 398 or 399 of another γ-chain polypeptide.

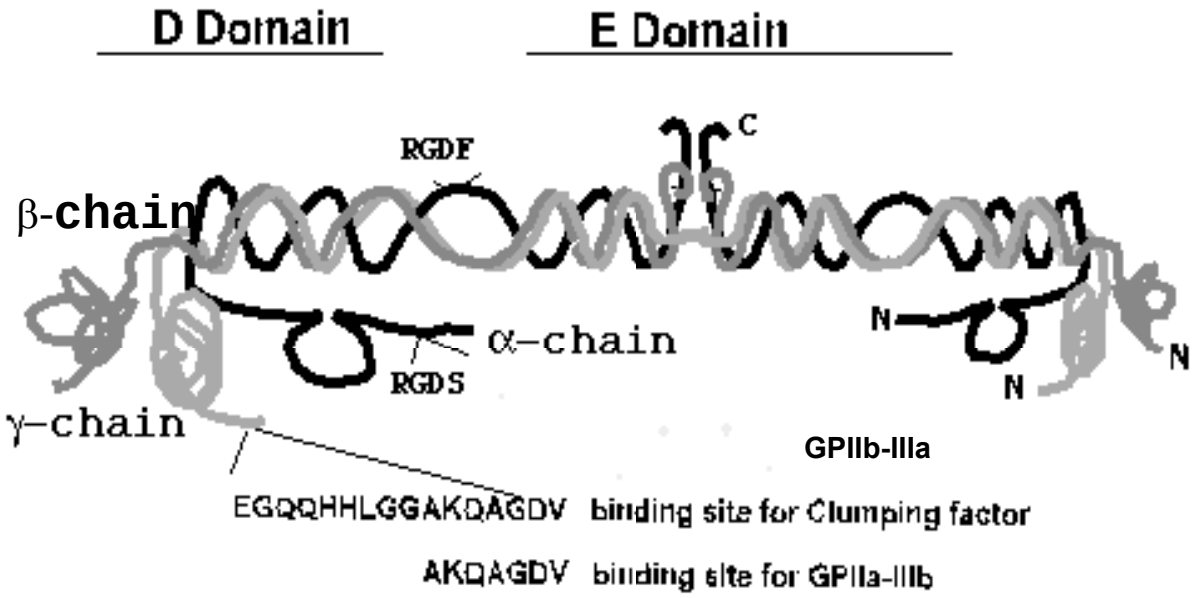


Figure 3. Schematic drawing of fibrinogen. The dimeric molecule is composed of three polypeptide chains, α, β and γ. The binding sites for ClfA and GPIIb-IIIa are indicated on the fibrinogen molecule (including the RGD sequences).

Platelet aggregation is a primary event in hemostasis and thrombus formation. Fibrinogen and fibrin contain several potential binding sites that interact with platelet GPIIb/IIIa receptors (31). One binding site (sequence HHLGGAKQAGDV) is located at the carboxy-terminus of the fibrinogen γ-chain (47). Other potential platelet GPIIb/IIIa binding site in Fg contain an Arg-Gly-Asp (RGD) sequence in the Aα95-97 and Aα572-574 regions (78). However, the Aα-chain carboxy-terminus (Aα572 to 574) is not conserved between species as expected for a potential binding site, but the RGD at position Aα95 to 97 is conserved between species. Since the RGD - containing peptide found in certain snake venoms is a powerful inhibitor of the fibrinogen-binding to platelets, at least one RGD site is essential for the Fg interaction with GPIIb-IIIa (30).

Platelet aggregation can be reversible or irreversible. Reversible aggregation is mediated by the binding of fibrinogen to GPIIb-IIIa which occurs in the absence of secretion of the contents of platelet storage granules. In contrast, irreversible aggregation is secretion dependent. Thrombospondin is released in significant amounts by activated platelets and plays an important role in the stabilization of the fibrinogen platelet receptor interactions. Thrombospondin mediates irreversible platelet aggregation by binding to receptors on

platelets and to fibrinogen which is bound to glycoprotein GPIIb-IIIa. Thrombospondin binds specifically to Fg with a K_d of 3.4 nM. Thrombospondin binding sites on Fg are located at residues 113 to 126 of the Aα-chain and residues 243 to 252 of the Bβ-chain (4).

It is well known that calcium is required for fibrinogen function since fibrin formation in the absence of calcium is significantly weaker then when it is formed in the presence of calcium. Three high affinity calcium binding sites have been determined for fibrinogen which have dissociation constants of K_d of 2×10^{-6} M (59). At least one of these is located at the C-terminal region of the γ-chain which contains a putative sequence resembles an EF-hand, Ca^{2+}-binding motif (DNDNDKFEGNC). Another site was identified on the Aα-chain. Fibrinogen with an intact Aα-chain possesses three high-affinity binding sites but fibrinogen samples with partially degraded Aα-chain exhibited only two sites (58).

AIMS

The aim of this study was to investigate FgBPs from *Staphylococcus aureus*, with the main focus on Efb (previously Fib) and on a 60 kDa fibrinogen- and prothrombin-binding protein.

The specific aims were to:

Construct an isogenic mutant deficient in Efb protein for subsequent comparison with the wild type in a rat infection model.

Identification of fibrinogen-binding sites on Efb and the regions on fibrinogen recognised by Efb.

Characterization of the 60 kDa fibrinogen- and prothrombin-binding protein.

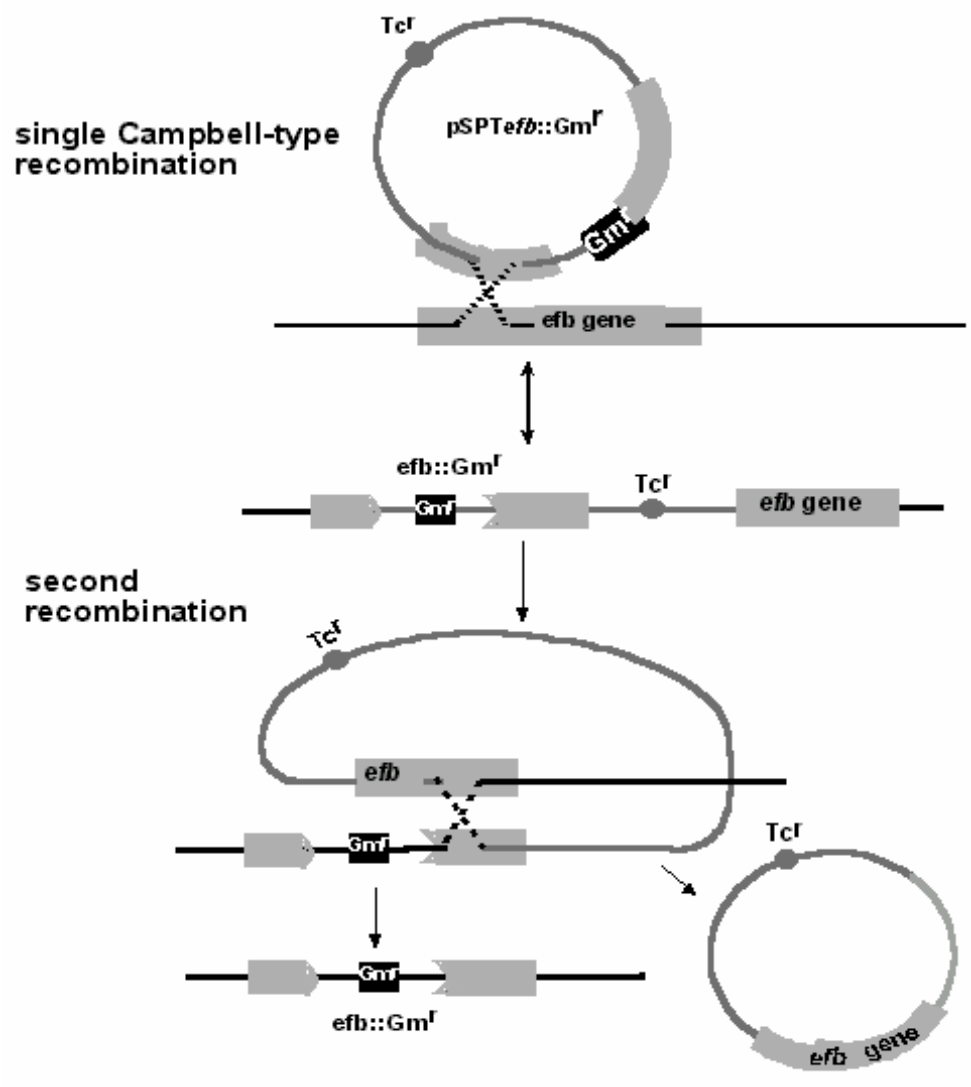
METHODS

Insertional inactivation of efb gene

The use of mutations in genetic research are important tools for the determination of the specific functions of virulence factors. A mutation which is an alteration in the nucleotide sequence of DNA molecule is a spontaneous event in nature. In bacteria one mutation occurs spontaneously for every 10^8 cell divisions. Different methods have been developed for the construction of mutants and for the isolation of organisms carrying the defective genes of interest. For instance a mutation in a bacterial cell can be induced when the cells are exposed to chemical mutagens. This can result in a large number of mutants including a few with mutations in the desired genes. This approach has been used in several bacteriological studies where the chemical mutagens have affected more than one gene. Of course, such results are difficult to interpret because of the large number of nonspecific effects. For the construction of site-specific mutations in a single gene, better methods are now available such as allele-replacement, transposon mutagenesis, plasmid integration and bacteriophage conversion.

Allele replacement was used in study **I** for the construction of an isogenic mutant defective in the gene encoding the extracellular fibrinogen-binding protein, Efb. In principal, allele replacement consists of insertion of a DNA fragment encoding drug resistance into the gene for the virulence factor of interest. The point is to disrupt the integrity of the virulence gene with the DNA fragment. The inserted fragment blocks gene transcription and the drug resistance is used as a marker for the identification of the mutated cells. In our study, a fragment from transposon Tn*4001* was used which contained the genes for gentamicin, kanamycin and tobramycin resistance. The *efb* gene was disrupted by this fragment in an unique *Xba*I site located upstream from the region responsible for fibrinogen-binding. Allele replacement consist of a substitution of the wild-type allele with the mutated plasmid-located copy. One way to do this is by a double recombination between homologous sequences; for example between a mutated (*efb*::Gm^r) and a wild-type copy of the *efb* gene.

Techniques and strategies on how staphylococcal isogenic mutants can be constructed have been described before. A suitable shuttle vector for this purpose which was used in this study is the vector pSPT181 which consist of the plasmids pSP64 and pRN8103. pRN8103 contains a determinant for temperature sensitive replication in *S. aureus* (a defect ORI) and the tetracycline resistance marker. pSP64 contains an ampicillin-resistance marker and origin of replication for *E. coli*. This shuttle vector replicates in Gram-negative organisms such as *E. coli* and in a Gram-positive organisms like *S. aureus*. Replication of pSPT181 plasmid occurs in staphylococci at the permissive temperature (30^oC), but not at the nonpermissive temperature (44^oC).



When the shuttle plasmid with the mutated *efb* gene has been established at the permissive temperature in the presence of antibiotic, it is possible to find a mixture of cells whose plasmid are replicating autonomously (not integrated) and cells where the plasmid has been integrated into the chromosome. Integration occurs by a single Campbell-type recombination although recombination occurs only at one site of the *efb* gene. Such strains are not suitable for virulence studies because one copy of the gene is still intact. Allele-replacement occurs when a second recombination takes place on the other side of the *efb* gene. This event causes the loss of the integrated plasmid containing a copy of the intact *efb* gene while the mutated gene is retained in the chromosome. This recombination occurs at a low frequency and requires a special approach to select cells which carry the defective *efb* gene (Figure 4).

Figure 4. Strategy used for the construction and selection of an isogenic mutant defective in the *efb* gene.

I. *Selection for single recombination*. First, the vector pPST181 containing the *efb*::Gm^r copy replicate autonomously in *S. aureus* at the permissive temperature (32^{o}C) in the presence of gentamicin and tetracycline. Under these conditions, the plasmid integrates into the chromosome by a single Campbell-type recombination in approximately 6 % of the cells, while the plasmids in the majority of the cells remain in an autonomous form. Cells with integrated plasmids may also have autonomously replicating plasmids.

II. *Selection for a double recombination*. Cells with an integrated vector contain two copies of the *efb* gene on the chromosome (the mutated and the wild type one). A second recombination takes place between the two *efb* genes when the bacterial cells are grown at 43^{o}C in the presence of gentamicin. Depending on which side of the *efb* gene the first recombination had taken place, the plasmid excised from the chromosome will have either an intact or a mutated *efb* gene.Thus, those cells that have lost the *efb*::Gm^r copy from the chromosome or those cells whose plasmid has never been integrated into the chromosome will be eliminated at 43^{o}C in the presence of Gm. These cells are unable to survive the antibiotic exposure since their plasmid containing the Gm^r gene can not be replicate at this temperature. Conversly, those cells where the recombination has take place at the other side of the *efb* gene, lose the intact copy of the *efb* gene but retain the *efb*::Gm^r in the chromosome.

III. *Amplification of the recombinant*. The proportion of cells that carry an integrated plasmid can be reduced during this step, at 43^{o}C without antibiotic. Mutants defective in the *efb* gene can be now detected by replica plating on plates with Gm or Tc, since these mutants are gentamicin resistant and tetracycline sensitive.

Isolation of extracellular FgBPs from S. aureus

Three extracellular fibrinogen-binding proteins have been reported previously by Bodén and Flock. They described an 87 kDa coagulase produced mainly during the exponential growth phase which was later replaced by a 60 kDa fibrinogen- and prothrombin - binding protein during the post-exponential growth phase. The third protein identified was described as a 19 kDa FgBP constitutively produced by *S .aureus* which they called Fib. In later studies the name Fib was changed to Efb for extracellular fibrinogen binding protein (**II**) since in the litterature Fib is sometimes used as an abbreviation for Fg.

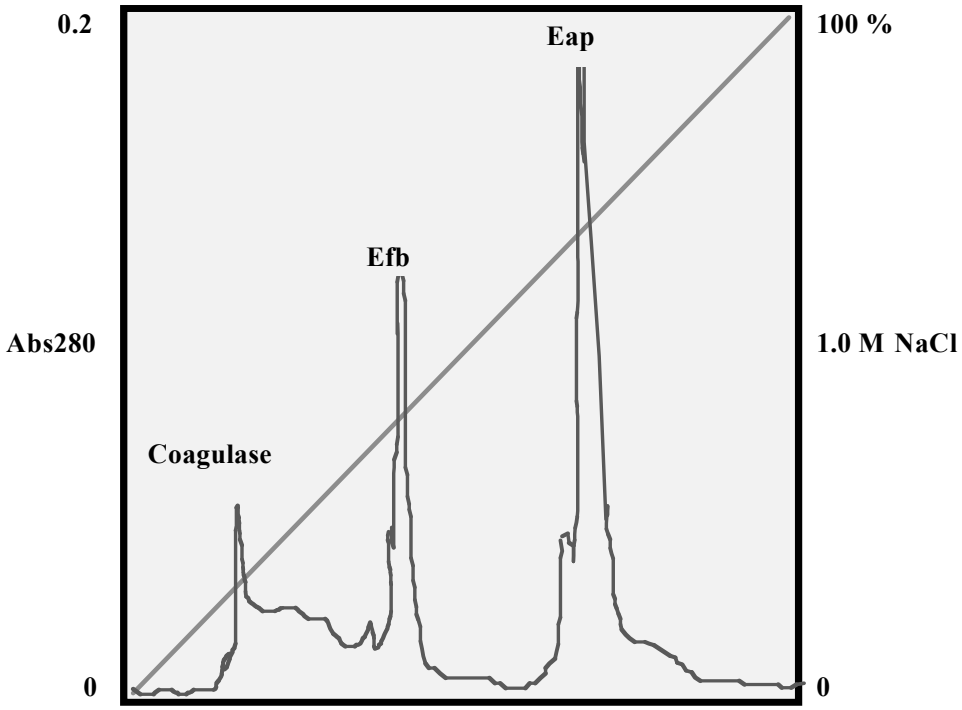


Figure 5. Separation of the extracellular fibrinogen-binding proteins from *S. aureus* using FPLC on a Mono-S column. The FPLC profile revealed three peaks corresponding to the three FgBPs

First, the fibrinogen-binding proteins were isolated from the culture supernatant of *Staphylococcus aureus* strain Newman by affinity chromatography on fibrinogen-Sepharose. In studies **II** and **V** a further purification step was added consisting of ion exchange chromatography which was necessary for the separation of three FgBPs. A Mono-S column coupled with Fast protein liquid chromatography (FPLC) equipment was used and the three proteins were eluted using an NaCl gradient (Figure 5). The first peak contained a 60 kDa

degradation product of the 87 kDa coagulase. It has been also demonstrated before that the repeats from the C-terminal region of coagulase are sensitive to degradation. The second protein eluted was Efb and the third was a protein which binds both to Fg and Pt but lacks coagulase activity. This protein was called Eap for extracellular adherence protein.

Isolation of the Aα–, Bβ– and γ– chains of fibrinogen

The most common method used for isolation of the chains of fibrinogen is the fractionation of the three polypeptides by ion exchange chromatography on CM-cellulose after reduction of the interchain disulfide bridges. In study **IV**, we developed a new method for separation of the polypeptides chains. This method consists of continuous-elution electrophoresis with a Model 491 Prep cell from Bio-Rad. Samples containing fibrinogen migrate electrophoretically through a 10 cm long cylindrical gel of 10% polyacrylamide. As the three chains migrate through the gel matrix, they are separated according to their molecular weight as with conventional gel electrophoresis. When individual bands migrate to the bottom of the gel, they pass directly into the elution chamber for collection. Purified molecules are drawn up through the elution collection tube by a peristaltic pump to a fraction collector.

Digestion of fibrinogen by plasmin

Proteolytic fragmentation of fibrinogen with plasmin has been used intensively in studies for the localization of functionally important sites on the fibrinogen molecule (**IV**). Digestion of Fg with plasmin yields a set of fragments designated A-E, according to their order of elution from a DEAE-cellulose column. Fragments A-C comprise a group of smaller peptides released during the course of digestion. Fragment D and E are the largest fragments generated during complete plasmid digestion of Fg (Figure 6). These fragments have masses of 50% and 15% of the fibrinogen molecule, respectively. Fragment D corresponds mainly to the distal domain and fragment E to the central domain. In addition, partial digestion of Fg with plasmin yields two intermediate fragments, X and Y. Fragment X is formed upon release of the carboxy terminal portion of the Aα-chain and the amino portion of the Bβ-chain. In this case the two distal domains of Fg remain connected by the central domain. But in fragment Y, only one distal domain is connected to the central domain.

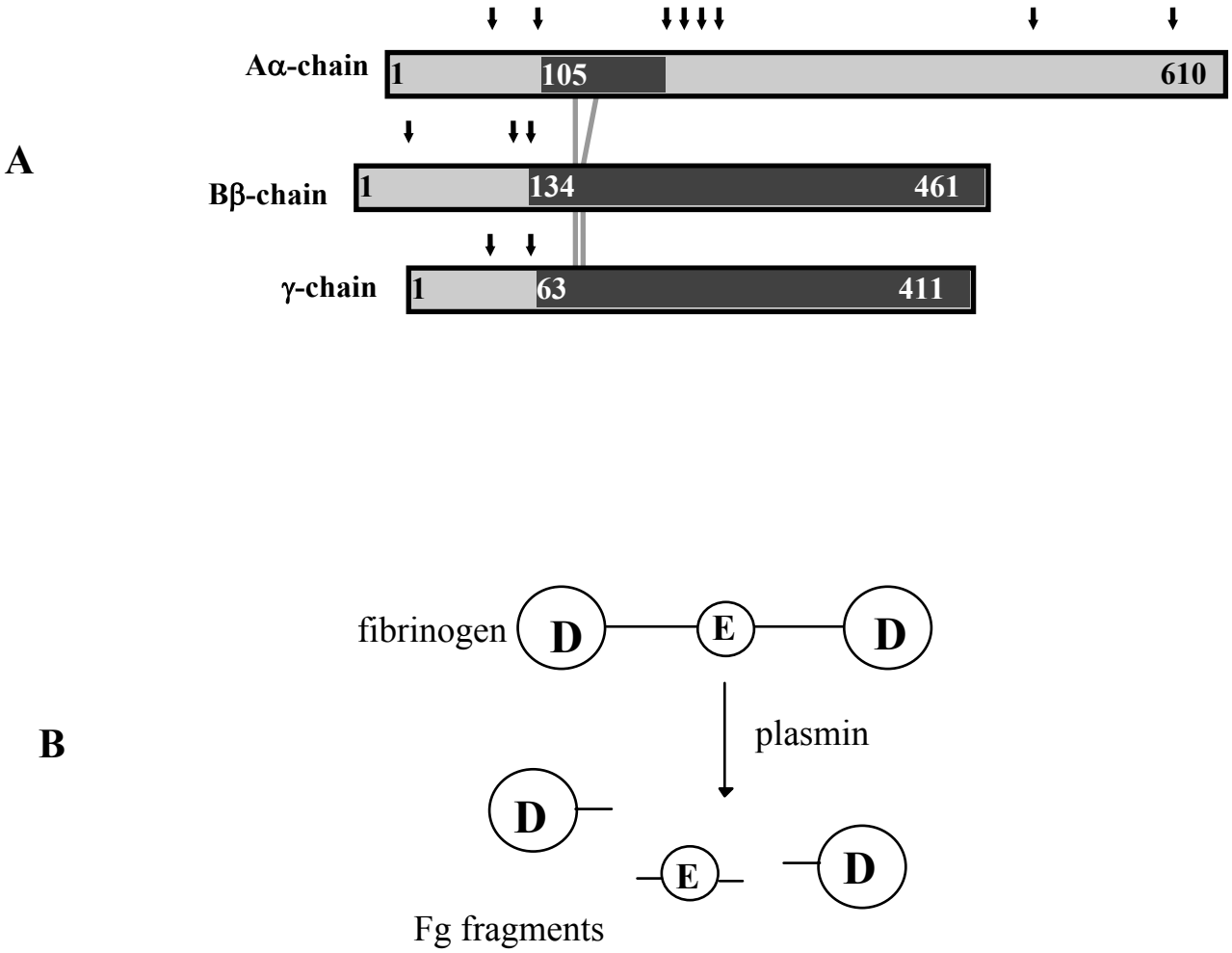


Figure 6. Schematic representation of plasmin- digested fibrinogen . A) The three polypeptides chains of Fg with respective cleavage sites for plasmin. The dark areas represent the regions including in the D fragment of fibrinogen. B) Fragment D and E form after plasmin cleavage.

RESULTS AND DISCUSSION

There are several secreted proteins from *S. aureus* that have affinity for fibrinogen, but their function are unclear since not all of them are involved in the bacterial attachment to the host. It is known that coagulase can convert fibrinogen to fibrin which can cover and protect staphylococci from the immune system. The function of the two remain extracellular fibrinogen-binding proteins (Efb and 60 kDa protein) have not been determined and are the basis of the present investigation.

Extracellular fibrinogen-binding protein (Efb)

That Efb is a potential virulence factor is suggested by data showing that 1) the incidence of the *efb* gene and its protein is around 100% among clinical isolates (12), 2) this protein is produced in vivo (17) and 3) we have constructed an isogenic mutant defective in the *efb* gene to analyse the virulence of *S. aureus* in wound infections in the presence or absence of the Efb protein (**I**). With an experimental wound infection model in rats, we demonstrated that Efb contributes to the virulence of *Staphylococcus aureus* in this model. We also showed that twice as many animals challenged with the parenteral strain developed severe clinical signs of wound infection compared with those infected with the isogenic mutant. The different levels of severity between these two groups also resulted in differential rates of weight loss. These results support previous data from a mouse mastitis model which was used to study the effect of vaccination with FgBP against staphylococcal infection (57). Vaccination with Efb reduced the rate of mastitis compared with controls vaccinated with collagen-binding protein. Since the mammary glands were traumatized during the procedure, this model mimics not only mastitis but also resembles a wound infection caused by *S. aureus.* A protective effect of anti-Efb antibodies against wound infection is therefore likely.

The fact that the antibody response to Efb was significantly lower in patients with staphylococcal infection compared to the normal population suggests that Efb may be a suitable agent for a vaccine against staphylococcal infection (17). It is also possible that passive immunization using anti Efb antibodies may provide protection against staphylococcal infection in patients with high susceptibility to infection as in immunocomprimised patients undergoing surgery.

The exact biological function of Efb in wound- or other infections is unclear, but it could be related to Fg-binding since fibrin is abundant in wounds and fibrinogen-/fibrin-binding is the only known function of Efb. The function of Efb is not related to the adherence of the bacteria to fibrinogen because Efb is an extracellular protein and the ability of an *efb* negative mutant

to bind to immobilized Fg is unaltered (**I**). Probably, the binding of Efb to functional sites on fibrinogen disturbes the healing process which could be beneficial for the bacteria (**I**).

The mechanism of action of Efb can only be speculative, but we have demonstrated that there are multiple binding sites involved in the binding of Efb to fibrinogen (**II**). With two different fusion proteins containing different regions of the Efb molecule, it was shown that one binding site is located at the C - terminal region of Efb and the other binding domain is located at the two repeat region of the N - terminal portion of the molecule (Figure 7). This finding was later confirmed using synthetic peptides corresponding to different regions of the Efb molecule (**III**)(Figure 8). Both peptides from the C-terminal portion of Efb (residues 91 - 136) and peptides containing the second repeat of the N-terminal region of Efb bind strongly to soluble fibrinogen and they inhibit the Efb-fibrinogen interaction. Therefore, a singel Efb molecule is able to bind two fibrinogen molecules simultaneously. When these two proteins are mixed in equimolar concentrations, the Efb-fibrinogen complex precipitates (**II**). Whether or not such precipitation occurs in vivo and its possible functional significance are unknown. It should be re-emphasized that Efb is not coagulase and the precipitation of the Efb-Fg complex is different from coagulation caused by staphylocoagulase. However, Efb and coagulase share a degree of sequence homology.

Paper **IV** shows that 1) the repeat region of Efb competes with the corresponding region of coagulase for the same binding domain on fibrinogen, 2) the binding of fibrinogen to an immobilized fusion protein containing the two repeats of Efb is blocked by the presence of coagulase and 3) the binding of Fg to immobilized coagulase is inhibited by the presence of Efb or its two repeat regions. The binding similarity of Efb and coagulase is related to the high degree of homology between these two proteins. We have found that the homology of the second repeat region of Efb and the repeat region of coagulase is greater than the homology of the first repeat of Efb , with the repeats of coagulase (82% versus 53%)(Figure 1). Moreover, a putative Ca^{2+} binding site has been predicted at the second repeat of Efb, at residues 51-58 (E-F-N-D-G-T-F-E), which resembles an EF-hand motif (**III**). Several human proteins bind to Ca^{2+}. Also proteins produced by *S. aureus,* such as Clf and Sdr can bind to Ca^{2+}.

The precipitation of the Efb-Fg complex from equimolar mixtures of these two proteins can be enhanced by the presence of Ca^{2+} or Zn^{2+}, but not by Mg^{2+} (**II**). The binding of fibrinogen to

immobilized Efb is increased by Ca^{2+} (**III**). Calcium is necessary for a number of biochemical

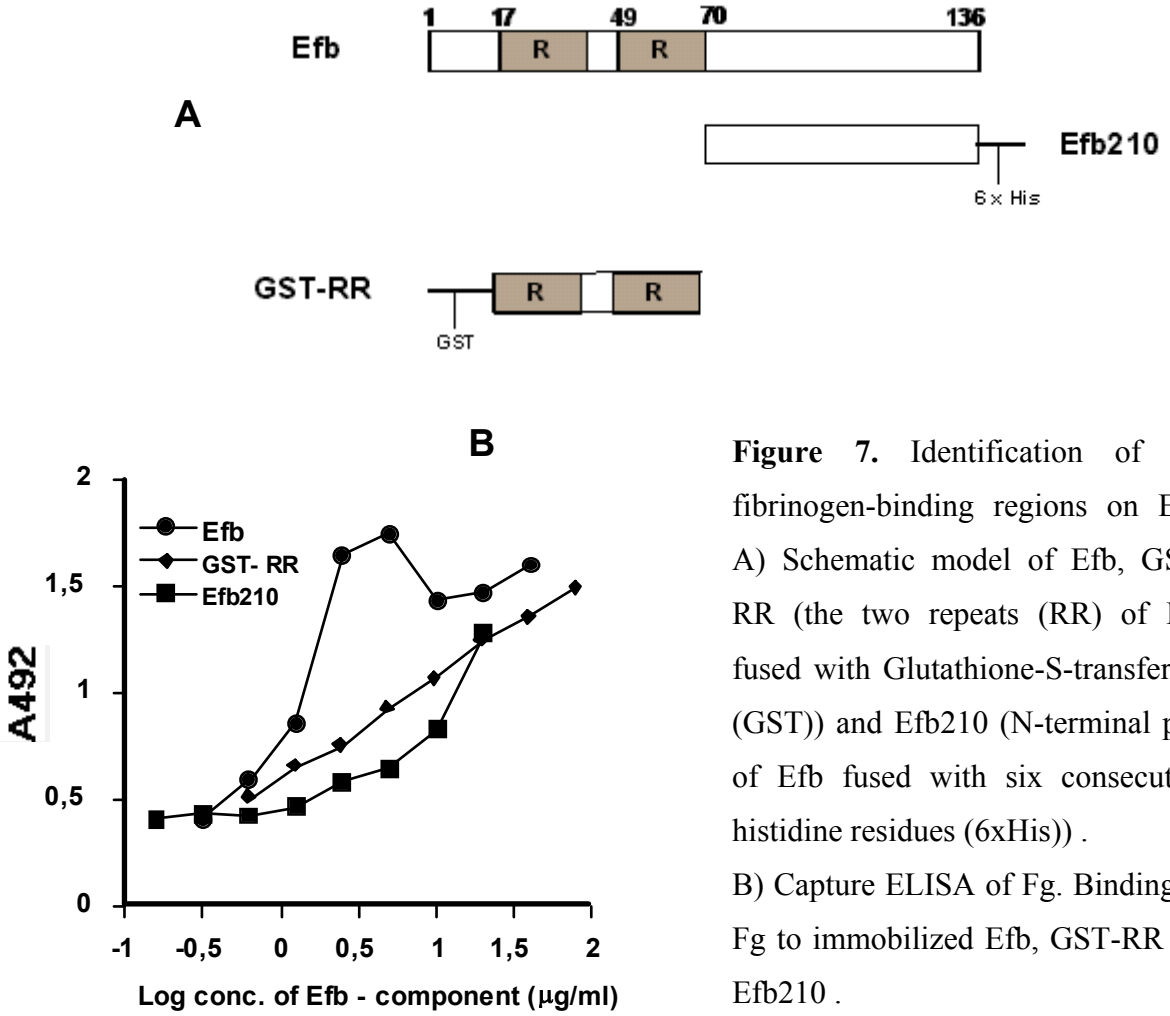


Figure 7. Identification of the fibrinogen-binding regions on Efb. A) Schematic model of Efb, GST-RR (the two repeats (RR) of Efb fused with Glutathione-S-transferase (GST)) and Efb210 (N-terminal part of Efb fused with six consecutive histidine residues (6xHis)) .
B) Capture ELISA of Fg. Binding of Fg to immobilized Efb, GST-RR or Efb210 .

processes such as the formation of blood clots. It is likely that Ca^{2+} influences the affinity of Efb for Fg during infections caused by *Staphylococcus aureus*.

In order to understand the function of FgBPs, it is important to know the binding epitopes on fibrinogen for staphylococcal proteins. This knowledge could lead to the development of new antistaphylococcal treatments and new vaccines. The binding domains for the different

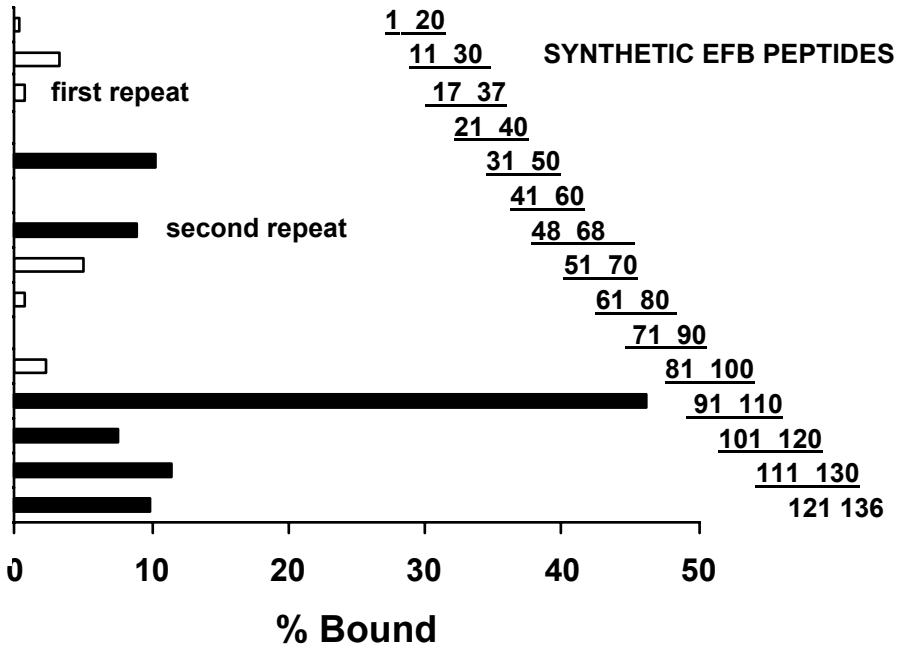


Figure 8. Binding of radiolabelled Efb peptides to soluble human fibrinogen (Wade *et al*., 1998)(**III**). The filled bars show the Efb peptides that bind to Fg to the greatest extent.

fibrinogen-binding proteins are distinct. Binding of ClfA takes place on the γ-chain of Fg, whereas ClfB binding takes place on both the Aα- and Bβ-chains. Efb does not interfere with the adherence of ClfA since ClfA inhibits up to 90% of the binding of Fg to immobilized Clf, while Efb has no effect (**II**). In our investigation we found that the Efb protein interacts with the Aα-chain of fibrinogen, indicating that at least one of the binding sites for Efb is located at the Aα-chain (**II** and **IV**). More detailed analysis showed that the D fragment obtained from plasmic cleavage of fibrinogen retained the same binding properties to Efb characteristic of the complete Fg molecule (**IV**). This indicates that Efb interacts with at least one of the polypeptides located on the D fragment. The D fragment includes three polypeptide fragments represented by bands on SDS-PAGE of 45 kDa for the Bβ-chain, 42 kDa for the γ-chain and a 14 kDa polypeptide from the Aα-chain. The Efb binding site on Fg is located between residues 105 and 197 of the Aα-chain of the D fragment. Binding of Efb to sites of functional importance on Fg may interfere with important physiological processes such as wound healing. Interestingly, several important functions have been attributed to the Aα- polypeptide chain of the D fragment or nearby regions. These functions that may be disturbed by the binding of Efb.

i) An Arg-Gly-Asp (RGD) sequence which is conserved among several mammalian species is located at residues 95-97 (only some residues from the N-terminal end of the Aα-chain of the

D fragment) (30, 31). This RGD sequence is one of the regions on Fg recognised by platelet integrin GPIIb-IIIa. It is possible that Fg-GPIIb-IIIa binding is sterically hindered by the presence of the Efb. Another platelet binding site located at the C-terminal end of the γ-chain of Fg is recognised by ClfA but not by Efb. A recombinant protein containing residues 221-550 of ClfA inhibits fibrinogen-dependent platelet aggregation and platelet adhesion to immobilized fibrinogen. Interestingly, three short regions in the Efb sequence are similar to three different regions of the glycoprotein GPIIIa (Figure 9). This observation should be the focus of further investigations on the biological functions of Efb. None of the three regions of Efb or GPIIIa are involved in the fibrinogen-binding. We can not exclude, however, that Efb can interfer with other functions of integrin. For example, GPIIIa is present on endothelial cells as part of the vitronectin receptor.

GPIIIa $_{19}$**KPVS**$_{22}$----------$_{265}$**IVQPNDG**$_{271}$----------$_{609}$**TFKKE**$_{623}$
Efb $_{11}$**KPVS**$_{13}$----------$_{19}$**IVEYNDG**$_{25}$----------$_{75}$**TIKKE**$_{79}$

Figure 9. Similarity between GPIIIa and Efb.

ii) Thrombospondin is the most abundant protein component of the α-granula of platelets activated at the site injury. Thrombospondin binds to fibrinogen and to the platelet surface and thereby mediates irreversible platelet aggregation (91). Thrombospondin therefore helps to stabilize the adhesion of fibrinogen to activated platelets. One of the thrombospondin-binding sites is located at residues 113-126 of the Aα-chain of fibrinogen (4); a region that is included in the polypeptide recognised by Efb.

iii) Plasminogen activation is catalyzed by a tissue-type plasminogen activator (t-PA). Fibrin and some fibrinogen degradation products such as fragment D but not intact fibrinogen accelerate the activation of plasmin by t-PA. More detailed studies revealed that an essential role is played by Aα148-161 in this process (95). Binding of Efb to this region probably inhibits the activation of plasminogen by t-PA thus disturbing the fibrinolytic pathway.

Efb may also interfere with the conversion of Fg to fibrin or other functions of fibrinogen such as the interaction with endothelial cells and fibronectin.

In conclusion, Efb increases the virulence of *S. aureus* in wound infections. Efb blocks important functional sites on fibrinogen that may delay wound healing which could be beneficial for the bacteria.

Extracellular adherence protein (Eap)

In the second part of this thesis we characterized a 60 kDa fibrinogen-binding protein designated as Eap, extracellular adherence protein (**V**). In culture, Eap is a secreted protein since 70% of the total Eap is found in the growth medium compared with only 30 % on the cell surface. It is possible that the function of Eap is distinct from the function of other fibrinogen-binding proteins from *S. aureus* since Eap has a broader binding profile as indicated by the following observations (**V**):

1) At least seven proteins from human plasma are enriched by an Eap-Sepharose column, including, prothrombin, fibronectin and fibrinogen. However, Eap has a targeted specificity for these proteins since not every protein from plasma bind to Eap. Human serum albumin, collagen type II and lysozyme do not interact with Eap in a capture ELISA, or in a western blot.

2) Eap can form oligomers. Eap – Eap interactions were demonstrated by affinity chromatography using Eap-Sepharose where a 60 kDa protein was isolated from the supernate of an *S. aureus* culture. Morever, Eap spotted at various concentrations onto a nitrocellulose filter was detected by an iodinated Eap probe.

3) Exogenously applied Eap interacts with the surface of *Staphylococcus aureus*. In studies examining the interaction of *S. aureus* and immobilized Fg, we found that while CflA inhibited bacterial binding to fibrinogen, Eap actually enhanced binding. The stimulation of the staphylococcal adherence by Eap led us to investigate the direct interaction between *S. aureus* an Eap. This finding was confirmed and extended by studies showing that soluble iodinated Eap could bind to *S. aureus* in a dose-dependent manner and that staphylococcal cells could adhere to immobilized Eap.

The binding of an extracellular protein to the surface of bacteria was described previously in *Listeria monocytogenes. Listeria* expresses two kinds of internalin (InlA and InlB) which are required for bacterial entry into the host cell (13, 14). The surface-associated Internalin A has an LPTXG motif which anchors the protein to a peptidoglycan. But Internalin B which is mainly secreted lacks this motif. Like Eap, InlB binds to the bacterial surfaces when added exogenously (13, 14). Otherwise no other relationship between Eap and InlB has been noted. Eap also has the capacity to agglutinate bacterial cells. Due to its ability to form oligomers and to bind to cells of *S. aureus*, Eap was expected to cause aggregation. In fact, it has been

known for some time that *S. aureus* forms aggregates. Which components on the bacterial surface are recognised by Eap is still unkwon. The number of binding sites per cell is very high indicating that components other than a protein receptor may be involved.

The presence of Eap in solution stimulates the attachment of *Staphylococcus aureus* to epithelial cells and fibroblasts. It thus seems that Eap can act as a bridging molecule between the bacteria and the host cells. Which components of the host cell interact with Eap is unclear, but it is possible that plasma proteins bound to integrin receptors might be recognised by Eap. In this situation, Eap might recognise fibronectin bound to integrin $\alpha 5\beta 1$ on fibroblast cells.

The amino- and carboxy-terminal sequences of Eap were analyzed to find similarities with a known sequence. The amino terminal sequence of Eap has a high degree of homology with the N-terminal sequence of the Map protein from *S. aureus* strain FDA574, major histocompatibility complex class II analogous protein (Figure 10). While the carboxy terminal sequence of Eap is different from that of Map, the C-terminal sequence of Efb is identical to the C-terminal of Map from strain Newman (unpublished sequence, partial sequence EMBL accession nr. 4138456). Map has been described as a surface-associated protein lacking an LPTXG sequence (38). The percentage of the total Map produced by *S. aureus* that is located in the culture supernatant or on the cell-surface has not been described previously. Similar to Eap, Map has a broad binding profile and interactes with several plasma and matrix proteins (34).

A 100% homology was found between the amino- and carboxy-terminal sequences of Eap with the N- and C-terminal sequences of a 70 kDa protein previously described by Yousif and coworkers (p70) (24, 98) (unpublished sequence, EMBL, accession nr. Y10419). Furthermore, The entire amino acid sequence of p70 is highly homologous (74%) to the sequence of Map. The homology between the region located between residues 1 to 540 of Map and the corresponding region on p70 is 84% while the rest of the amino acid sequence shares only 27% similarity (Figure 10). This indicates that p70 is similar to Map and may belong to a family of related proteins. It is possible that Eap may be related to p70 and Map. The observation that Eap is secreted while the another proteins are not, may be a strain variation.

p70 belongs to a group of proteins described as highly cationic staphylococcal proteins due to their extreme isolectric point of 10 (100). p70 like a 32 kDa neutral phosphatase (NPtase) interacts with immunoglobulin without a specific antigen-antibody recognition. We have noted a nonspecific interaction between Eap and immunoglobulin during capture ELISA. It has been shown that both the 32 kDa protein and p70 bind to the glomerular basement

membrane (GBM) of rats. These proteins may be involved in glomerular injury in glomerulonephritis

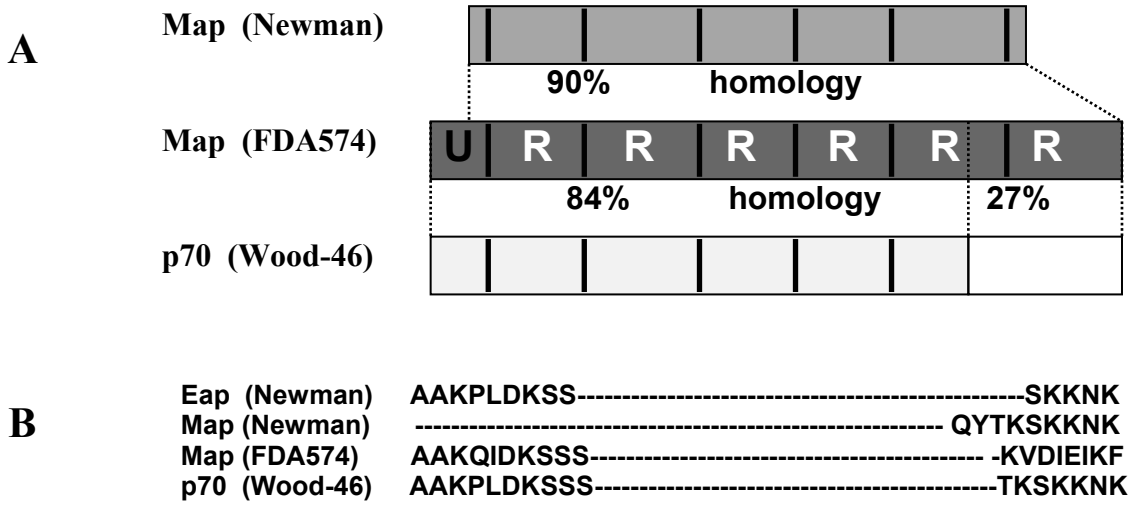


Figure 10. Similarity between Map, p70 and Eap. A) Schematic drawing of Map from *S. aureus* strain Newman and FDA574 and p70 protein from strain Wood-46. Map consist of a signal sequence (S) (not included in our picture) followed by a unique sequence (U) and several repeated domains (R). The percent homology between the sequences of Map from different strains are indicated. B) Comparison of the N- and C-terminal sequences of Eap, Map and p70.

(24, 99). This suggests that p70 (and Eap) has the ability to bind to the extracellular matrix located in the GBM.

In conclusion, we have characterized an extracellular protein that possesses both broad binding specificity to matrix and plasma proteins, and the capacity to recognize bacterial cell surfaces. We proposed a mechanism of adherence enhancement of *S. aureus* to host components stimulated by Eap.

SUMMARY

An extracellular fibrinogen-binding protein (Efb) from *Staphylococcus aureus*:
* contributes to the virulence of *Staphylococcus aureus* in wound infections
* binds to sites of functional importance on the Aα-chain of fibrinogen such as regions close to the Arg-Gly-Asp (RGD) sequence and to the thrombospondin-binding site. This may interfere with platelet binding to fibrinogen.
*contains two binding sites for fibrinogen-binding. One binding site is located at C terminus of Efb and the other is located at the two repeat regions of the N terminus.

An extracellular adherence protein (Eap) from *Staphylococcus aureus*:
* has a broad binding profile. Eap binds to at least seven plasma proteins, including fibrinogen, prothrombin and fibronectin.
* stimulates attachment of *S. aureus* to host cells. This may represent a novel mechanism for the adherence of *S. aureus* to host components.
* Is similar and may be related to Map and p70 protein from *S. aureus*. In contrast to Map and p70, Eap is secreted.

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